

Contrasting impairments in IgM and IgG responses of vitamin A-deficient mice

(immunity/T lymphocytes/B lymphocytes/immunoglobulins/retinoids)

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ABSTRACT Mice fed a semipurified, vitamin A-deficient diet (A⁻ mice) and control animals fed the same diet with added retinyl acetate (A⁺ mice) were used to investigate the effect of vitamin A deficiency on primary immunoglobulin responses to protein antigens. At age 6 weeks, A⁻ mice had serum retinol concentrations that were 46% of A⁺ controls. When immunized with a single antigen dose, these mice produced an antigen-specific IgM response equivalent to controls, but their IgG1 and IgG3 responses were sharply diminished (<30% of A⁺ controls). At age 8 weeks, A⁻ mice had 20% of A⁺ serum retinol concentrations and <17% of A⁺ liver retinyl palmitate levels. Responding to a single antigen dose, A⁻ mice produced ≈70% as much IgM as A⁺ controls. Their IgG1 response was <30% and their IgG3 response <3% of A⁺ controls. The IgG1 response kinetics were identical in A⁻ and A⁺ mice. Diminished serum antibody responses in A⁻ mice were attributable to fewer immunoglobulin-secreting plasma cells rather than to a decline in IgM or IgG secretion rate per cell. Total serum IgG3 levels, irrespective of antigen specificity, were slightly elevated in A⁻ mice compared to A⁺ controls. The inefficient clonal expansion of responding B lymphocytes and contrasting impairment of IgM and IgG responses observed in vitamin A-deficient mice are discussed with respect to a possible helper/inducer-T-lymphocyte defect.

Vitamin A supports vision, growth, reproduction, cellular differentiation, and immune function (1). It is also a potent chemotherapeutic agent against many tumors (1). The vitamin seems to modulate gene expression (1), perhaps as a hormone-like molecule or as a signal transducer. However, little is known about the mechanisms by which vitamin A exerts its biological functions in all but the visual cycle.

With respect to immunity, supplementary vitamin A enhanced delayed hypersensitivity (2), cytotoxic activity (3), and graft-versus-host responses (4) to antigens. Serum immunoglobulin responses and plaque-forming cell numbers were also increased by vitamin A supplementation (5–7). Accompanying immune enhancement were elevated Lyt-1⁺ T-lymphocyte numbers (4) and interleukin-2 production (8). Immune enhancement occurred only when the vitamin supplement preceded antigen administration (5, 9).

Conversely, vitamin A deficiency is associated with depressed immunity. Delayed hypersensitivity to a contact allergen (10), natural killer cell activity (11), and mitogen responses (12) were depressed during vitamin A deficiency. Similarly, decreased serum antibody responses occurred in animals with reduced serum retinol concentrations (10, 13–19). Excepting Sirisinha *et al.* (17), who studied IgA, and Smith *et al.* (10), who studied IgM, investigators have not analyzed the effect of vitamin A deficiency on individual immunoglobulin isotype responses. In addition, the response

kinetics have not been compared in vitamin A-deficient and vitamin A-sufficient animals.

To probe the cellular and molecular basis for the observed decline in cellular and humoral immunity, we developed a murine vitamin A deficiency model (10). Three vitamin A deficiency states were defined in mice fed a semipurified diet devoid of vitamin A. Vitamin A-deprived mice, age 6 weeks, showed a 54% decline in serum retinol compared to controls. Vitamin A-deficient mice, age 8 weeks, had an 80% decline in serum retinol without signs of inanition. Severe vitamin A deficiency occurred together with inanition in 11-week-old mice.

We analyzed individual immunoglobulin isotype responses to new protein antigens. Results reported here show a striking differential effect of vitamin A deficiency. In vitamin A-deficient mice, the IgG1 and IgG3 responses were impaired earlier and to a greater extent than were IgM responses. The IgG response kinetics, like IgM response kinetics (10), were unaltered. Clonal expansion of antigen-responsive B cells was less efficient in vitamin A-deficient mice. We suggest that a functional helper-T-cell defect may account for our observations.

MATERIALS AND METHODS

Vitamin A-Deficient Mice. Vitamin A-deficient mice were produced as described (10) except that the diet was modified slightly; the diets used in this work are shown in Table 1. The B10.BR mice were from The Jackson Laboratory, and B10.LG breeding pairs were from F. Bach (Department of Pathology and Laboratory Medicine, University of Minnesota). Animals were housed as described (10).

Chemicals and Media. Hen egg-white lysozyme, *p*-nitrophenyl phosphate, *p*-nitrophenyl β -D-galactopyranoside, Tween-20, and avidin-alkaline phosphatase were purchased from Sigma, and keyhole limpet hemocyanin and biotinyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester were from Calbiochem. Avidin- β -galactosidase was from Bethesda Research Laboratories. GIBCO supplied culture media and supplements; fetal bovine serum was from HyClone (Logan, UT).

Retinoid Analysis. High-performance liquid chromatography (20), as modified by Smith *et al.* (10), was used to quantitate serum retinol. Liver retinyl palmitate was extracted according to published methods (21, 22); retinyl acetate (50 μ g/g of liver) was the internal standard. Ether extracts were evaporated to dryness under N₂, redissolved in chloroform/methanol (2:1, vol/vol), and centrifuged (1000 \times g, 10 min). A supernatant aliquot (200 μ l) was evaporated and redissolved in 1 ml of acetonitrile, and 30 μ l was injected into the HPLC column as described (10). A 1%/min ethyl acetate gradient into acetonitrile (1.5 ml/min) eluted the retinoids. Retinyl acetate was eluted at 3% ethyl acetate, retinyl palmitate was eluted at 28% ethyl acetate.

Antibodies. Goat antibodies to mouse κ light chain, to IgM, to IgG1, and to IgG3 immunoglobulins were from Southern

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Table 1. Mouse diet

Ingredient	Concentration
	(% by weight)
Dextrose monohydrate	44.08
Sucrose	21.71
Casein	18.00
Amino acid supplement*	1.05
CaCO ₃	2.18
KH ₂ PO ₄ + K ₂ HPO ₄	0.90
NaH ₂ PO ₄	1.19
Cellulose	3.00
Choline chloride	0.20
Sodium ascorbate	0.10
Water-soluble vitamins†	0.20
Salt mixture‡	2.39
Cottonseed oil	5.00
	(μg per day)
α-Tocopherol	293
Menadione	35
Ergocalciferol	1.3
Retinyl acetate	25 (A+ diet only)

This experimental diet, modified from ref. 10, meets or exceeds requirements established by the American Institute of Nutrition. Breeder diet included 12.5% cottonseed oil and 36.58% dextrose monohydrate.

*Percent by weight: arginine, 23; glycine, 43; methionine, 15; cysteine, 19.

†Percent by weight: thiamine, 0.5; riboflavin, 0.5; pyridoxine, 0.5; pantothenate, 2.8; nicotinamide, 2.0; inositol, 20.0; folic acid, 0.10; vitamin B₁₂, 0.002; biotin, 0.01; glucose monohydrate, 73.6.

‡Percent by weight: KCl, 48; NaCl, 17.5; MgSO₄·7H₂O, 30.0; FeSO₄·7H₂O, 2.7; CuSO₄·5H₂O, 0.10; NaF, 0.094; CoCl₂·6H₂O, 0.003; KI, 0.008; MnSO₄·H₂O, 0.007; ZnSO₄·7H₂O, 0.55; (NH₄)₆-Mo₇O₂₄·4H₂O, 0.004; CrCl₃, 0.003; Na₂SeO₃, 0.001.

Biotechnology Associates. Litton Bionetics provided murine myeloma proteins. Rabbit antibodies to mouse IgM were purchased from Zymed Laboratories (Burlingame, CA). Peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were from Cappel Laboratories (Cochranville, PA). We coupled antibodies to biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester according to Guesdon *et al.* (23). Reagents were tested for specificity and used at optimal concentrations.

Serum Immunoglobulin Analysis. We immunized mice i.p. with 100 μg of antigen in buffered saline (50 mM phosphate, pH 7.2/0.15 M NaCl) and analyzed the antigen-specific serum immunoglobulin response in an enzyme-linked immunosorbent assay (ELISA; ref. 10). Antibodies to individual immunoglobulin isotypes used in the ELISA exhibited ≤5% crossreactivity with other isotypes. To quantitate total serum immunoglobulins, irrespective of antigen specificity, wells were coated with the diluted mouse serum sample or purified myeloma protein standards and the ELISA was completed as described. To quantitate total serum immunoglobulins, irrespective of heavy-chain isotype, goat antibodies to mouse κ light chains were used in the ELISA.

Immunoglobulin-Secreting Cells. We immunized mice with hemocyanin (100 μg i.p. in buffered saline) and enumerated immunoglobulin-secreting B cells by the filter immunoplaque assay exactly as described (24). In the absence of antigen, we observed 467 ± 238 IgM plaque-forming cells (PFC) per spleen and 0 ± 0 IgG1 PFC per spleen; these values were subtracted from experimental values. Nonimmune splenocytes yielded 962 ± 529 IgM PFC per spleen and 0 ± 0 IgG1 PFC per spleen on hemocyanin-coated filters.

Immunoglobulin Synthetic Rate. A fraction of the splenocytes from the plaque assay were tested for antigen-specific immunoglobulin secretion. Immune or nonimmune splenocytes (3.8–30 × 10⁶ cells per ml; 0.1 ml) were incubated on

nitrocellulose filter discs (Millititer HA plate; Millipore) that had been coated with hemocyanin (100 μg/ml) and blocked with nonfat dry milk (5% wt/vol) in buffered saline (50 mM phosphate, pH 7.2/0.15 M NaCl). Nitrocellulose discs without hemocyanin and blocked with nonfat milk served as background controls. After a 3-hr incubation (37°C, humidified 7.5% CO₂ in air), discs were washed extensively with buffered saline containing Tween-20 (0.05% vol/vol). Biotin-conjugated goat antibodies to mouse IgM (1 μg/ml) or IgG1 (2 μg/ml) were added; plates were incubated (4 hr, 23°C) and washed. Avidin conjugated to β-galactosidase (1:1000) was added. After 90 min at 23°C, discs were washed, punched out, and incubated in *p*-nitrophenyl β-D-galactopyranoside (1 mg/ml in 50 mM phosphate buffer, pH 7.2/1.5 mM MgCl₂). Finally, discs were removed and the absorbance at 405 nm was measured. Myeloma protein standards were run in parallel to quantitate bound immunoglobulin. Nonimmune mice yielded no detectable hemocyanin-specific immunoglobulin IgM or IgG1.

Statistical Analysis. Three to ten mice comprised each experimental group; experiments were repeated three times. The mean and standard deviation for replicates from each animal were used to calculate a mean and standard error of the mean for the group. Values for A– and A+ experimental groups were compared and the significance of differences was analyzed using the nonparametric Wilcoxon test (25).

RESULTS

The animals were produced according to Smith *et al.* (10). For mice fed a semipurified, vitamin A-deficient diet (A– mice), serum retinol was 46% of the A-sufficient (A+) control value at age 6 weeks and 20% of A+ control at age 8 weeks (Table 2). Liver retinoid stores of A– mice were also depleted at age 8 weeks; A– liver retinyl palmitate concentration was <17% of A+ controls. These A+ and A– animals showed no signs of inanition; their body weights were matched and they consumed the same amount of food per day (10). Thus, results reported here can be attributed to vitamin A deficiency.

We studied the IgM, IgG1, and IgG3 responses to two model protein antigens, hemocyanin and lysozyme (Table 3). In A– mice (age 6 weeks), the IgM response to hemocyanin was not significantly different from controls. However, the IgG1 response was <30% and the IgG3 response <20% of A+ control responses. As the deficiency progressed, IgM responses also declined. At age 8 weeks, A– mice produced only about 70% as much IgM as controls in response to hemocyanin. The IgG1 response of A– mice was still <30% of A+ controls and the IgG3 response was nearly undetectable. Serum antibodies to hemocyanin in unprimed A+ and A– mice were <10 μg/ml. The immune responses to lysozyme were weaker than the responses to hemocyanin, and no IgG3 response to lysozyme was detected (Table 3). The A– mice produced 44% as much IgM to lysozyme as control mice and their IgG1 response was 32% of A+ controls.

Table 2. Retinol concentrations and weights of A– and A+ mice

Age, weeks	Vitamin A status	Body weight, g	Serum retinol, μg/dl	Liver retinyl palmitate, μg/g
6	A+	18.7 ± 0.9	35 ± 5	ND
	A–	18.5 ± 0.8	16 ± 3	ND
8	A+	19.4 ± 2.2	41 ± 9	133 ± 21
	A–	19.4 ± 1.6	8 ± 4	22 ± 8

Retinoid concentrations were determined as described in *Materials and Methods*. Results represent the mean ± SD of 10–15 B10.LG mice per group. ND, not determined.

Table 3. Immunoglobulin responses to protein antigens

Age, weeks	Vitamin A status	Antigen	Immunoglobulin isotype, $\mu\text{g/ml}$		
			IgM	IgG1	IgG3
6	A+	Hemocyanin	240 \pm 30	1460 \pm 340	21 \pm 9
	A-	Hemocyanin	210 \pm 60	400 \pm 120	3 \pm 1
			(NS)	($P \leq 0.001$)	($P \leq 0.006$)
8	A+	Hemocyanin	140 \pm 10	920 \pm 130	112 \pm 28
	A-	Hemocyanin	100 \pm 10	260 \pm 50	3 \pm 1
			($P \leq 0.01$)	($P \leq 0.001$)	($P \leq 0.004$)
8	A+	Lysozyme	91 \pm 7	183 \pm 76	≤ 1
	A-	Lysozyme	40 \pm 11	58 \pm 17	≤ 1
			($P \leq 0.01$)	($P \leq 0.01$)	(NS)

Serum immunoglobulins were quantitated by ELISA 11 days after immunization. Results are expressed as the mean \pm SEM of three experiments with 5–10 mice per group. Mice primed with hemocyanin were pathogen-exposed B10.LG females; mice primed with lysozyme were pathogen-free B10.BR females. The significance of differences between the A+ and A- groups is shown parenthetically. NS, not significant.

The hemocyanin-specific serum IgG1 response kinetics were identical in A+ and A- mice immunized with a single antigen dose. The A- mice produced less IgG1 antibody than did A+ controls throughout the primary response (Fig. 1). The peak response for both groups occurred on day 15. From results presented in Table 3 and Fig. 1, we conclude that an early and dramatically decreased IgG1 and IgG3 response characterizes the vitamin A-deficient mouse. In addition, partially impaired IgM responses occurred later in vitamin A deficiency.

Diminished serum immunoglobulin responses could result from a paucity of immunoglobulin-secreting cells, a reduced antibody secretion rate per cell, or both. Therefore, we enumerated antigen-specific IgG1- and IgM-secreting B cells and quantitated antigen-specific IgM and IgG1 secretion rates in cells from A- and A+ mice (Figs. 2 and 3). The reduced number of antibody-secreting plasma cells per spleen correlated very well with the decline in serum immunoglobulin for both IgM and IgG1. In contrast, antibody secretion rates per cell did not differ significantly for splenocytes from A+ and A- mice. Evidently, diminished serum antibody responses are attributable to fewer immunoglobulin-secreting plasma cells in vitamin A-deficient mice, and not to a decline in secretion rate per cell.

Some studies (15, 26) report elevated serum immunoglobulin concentrations accompanying vitamin A deficiency. We measured total serum IgM, IgG1, and IgG3 (irrespective of antigen specificity) in unprimed A+ and A- mice (Table 4).

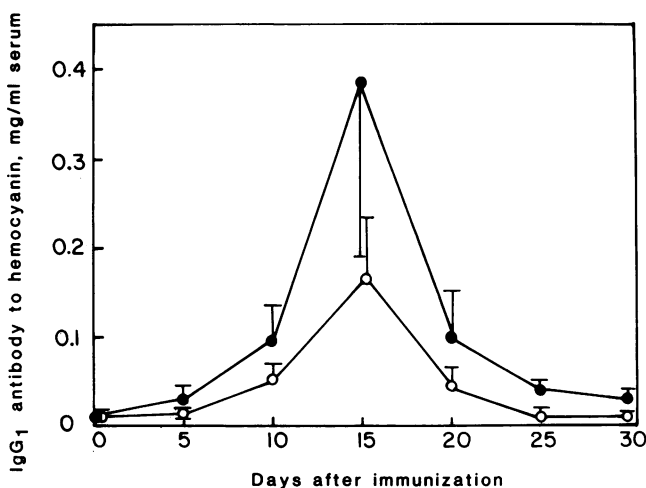


FIG. 1. Kinetics of the IgG1 response to hemocyanin in A- (○) and A+ (●) mice. Mice were B10.BR females, age 8 weeks, housed in a pathogen-free colony.

These were matched in mice at age 6 weeks (data not shown). Overall, the unprimed, pathogen-free animals had more serum IgM and less serum IgG than the pathogen-exposed animals. At age 8 weeks, IgG3 was increased almost 2-fold in the pathogen-free and pathogen-exposed A- mice compared to A+ controls, whereas their IgM concentrations were nearly identical. The pathogen-free A- mice had less serum IgG1 than controls. The total immunoglobulins with κ light chains were slightly elevated in pathogen-exposed, A- mice compared to controls, but pathogen-free A- and A+ mice did not differ. We conclude that a moderate increase in serum IgG3 characterizes the unprimed vitamin A-deficient mouse, irrespective of pathogen exposure.

DISCUSSION

Vitamin A deficiency impairs the ability of animals to resist infectious disease (27). Results presented here show that

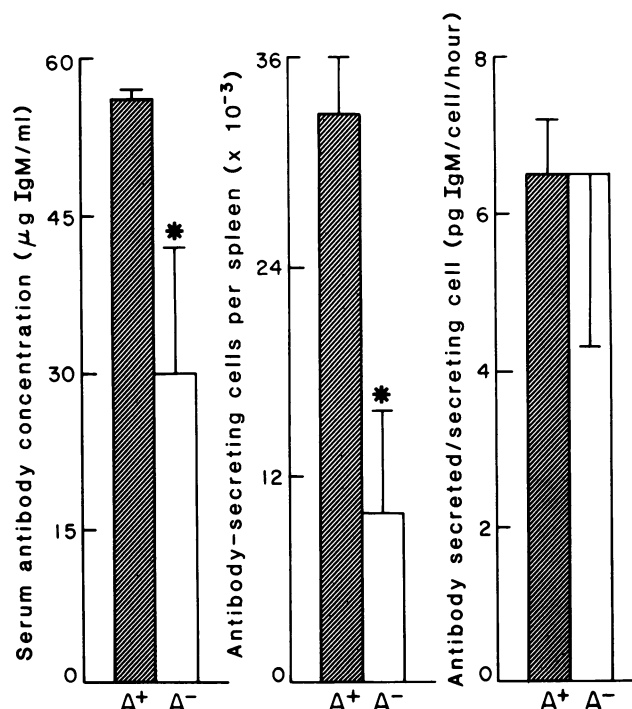


FIG. 2. Hemocyanin-specific IgM responses in A- and A+ mice immunized with hemocyanin and analyzed 5 days later. Significance was $P \leq 0.05$ (*). Mice were B10.BR females, age 8 weeks, housed in a pathogen-free colony.

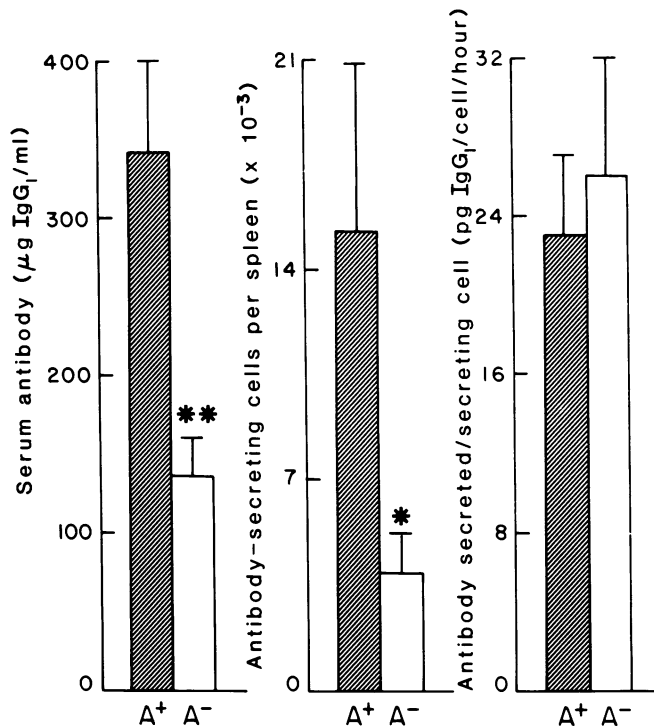


FIG. 3. Hemocyanin-specific IgG₁ responses in A⁻ and A⁺ mice immunized with hemocyanin and analyzed 11 days later. Significance was $P \leq 0.05$ (*) or $P \leq 0.001$ (**). Mice were B10.BR females, age 8 weeks, housed in a pathogen-free colony.

vitamin A deficiency affects IgM and IgG serum immunoglobulin primary responses quite differently. Vitamin A deficiency adversely affects IgG responses earlier and more drastically than IgM responses. Furthermore, we attributed the decline in serum immunoglobulin responses to fewer antibody-secreting cells rather than to a decreased secretion rate per cell. Decreased antibody responses could account in part for the inability of vitamin A-deficient animals to resist infection.

To our knowledge, the contrasting impairment of IgM and IgG immunoglobulin isotype responses accompanying vitamin A deficiency has not been reported previously. Our findings are consistent with other reports wherein antibody isotypes were not distinguished. Swine with reduced serum retinol levels (15) and chicks fed a diet with suboptimal vitamin A (28) showed reduced agglutination responses to

Salmonella pullorum bacterial antigens. Vitamin A-deficient rabbits (13) and rats (19) produced less serum hemolytic antibody when immunized with sheep erythrocytes. Finally, secreted intestinal IgA was reduced in rats cycled on a vitamin A-deficient diet followed by a retinoic acid-supplemented diet (17).

Although antibody responses to new antigens were lower, total serum immunoglobulin concentrations were slightly elevated in A⁻ mice exposed to pathogens (Table 4). Elevated concentrations of total serum immunoglobulins have also been observed in vitamin A-deficient swine (15), NZB mice (26), and rabbits (13). In contrast, other researchers reported normal levels of serum immunoglobulins in vitamin A-deficient rats (17). We tested pathogen-free, A⁻ mice; their total serum immunoglobulins matched controls. Thus, the dichotomy concerning total serum immunoglobulins in published reports can be attributed to differences in environmental pathogens to which animals were exposed.

The total serum immunoglobulin levels in vitamin A-deficient mice indicate that their B cells synthesize immunoglobulin quite well. Similarly, the IgM and IgG₁ secretion rates per secreting cell were unaffected by the deficiency. Although IgG₁ and IgG₃ antibody responses to new antigens were diminished in A⁻ mice, these isotypes were abundant in the serum of unprimed A⁻ mice. Therefore, B cells of A⁻ mice are not fundamentally defective in isotype-switching mechanisms. Our results show that the A⁻ mice developed fewer antigen-specific, antibody-secreting cells than did controls in response to immunization (Figs. 2 and 3). A reduced number of antibody-secreting cells was also observed in vitamin A-deficient rats (16, 19). Evidently, clonal expansion of responding B lymphocytes does not occur as efficiently in vitamin A-deficient mice as it does in controls, but vitamin A does not seem to influence B-cell antibody synthesis, secretion, or isotype switching.

Activated helper T lymphocytes promote clonal expansion of responding B lymphocytes, particularly IgG-secreting B cells. The lymphokine interleukin 4 supports B-cell clonal expansion and B-cell differentiation from IgM to IgG secretion (29, 30). This lymphokine is secreted by helper T lymphocytes responding to an antigenic stimulus. These T cells also secrete a second lymphokine, interleukin 2, which supports the growth of other antigen-responsive helper T cells (31). We hypothesize that vitamin A deficiency adversely affects helper-T-lymphocyte function, perhaps by blocking T-cell differentiation leading to the synthesis and secretion of one or more lymphokines required for a strong immune response.

The observation that A-deficiency affects IgG responses earlier and more dramatically than IgM responses is consistent with our hypothesis. IgM production is much less dependent on interleukin 4 than is IgG production (29, 30). We reported a decrease in T-cell-mediated delayed hypersensitivity (10) very early in vitamin A deficiency, a result that would be predicted if interleukin-2 synthesis and secretion were impaired in vitamin A deficiency. It is noteworthy that the T-cell-dependent responses, IgG secretion and delayed hypersensitivity, are decreased in the earliest stages of vitamin A deficiency, when serum retinol is only 50% of control values.

The vitamin A-deficient immune system resembles the nude mouse's immune system. The congenitally athymic nude mice, having few if any mature T lymphocytes, respond poorly to protein antigens. In unprimed *nu/nu* mice, total serum IgG₃ and IgM levels are elevated, whereas IgG₁, IgG_{2a}, and IgA levels are reduced compared to *nu/+* littermate controls (32). The similarities between nude and vitamin A-deficient mice support the hypothesis that vitamin A deficiency adversely affects helper-T-cell differentiation or function.

Table 4. Total serum immunoglobulins in unprimed A⁻ and A⁺ mice

Vitamin A status	Immunoglobulin isotype, µg/ml			
	IgM	IgG ₁	IgG ₃	Total
<i>Pathogen-exposed mice</i>				
A ⁺	215 ± 12	1340 ± 260	1740 ± 100	3715 ± 420
A ⁻	221 ± 14 (NS)	1420 ± 150 (NS)	3270 ± 414 ($P \leq 0.04$)	4723 ± 235 ($P \leq 0.04$)
<i>Pathogen-free mice</i>				
A ⁺	466 ± 41	889 ± 27	175 ± 22	2002 ± 144
A ⁻	430 ± 46 (NS)	505 ± 178 ($P \leq 0.05$)	307 ± 58 ($P \leq 0.05$)	1864 ± 563 (NS)

Serum immunoglobulins, irrespective of antigen specificity, in unprimed 8-week-old mice were quantitated by ELISA. Pathogen-exposed mice were B10.LG females; pathogen-free mice were B10.BR females. Results are expressed as the mean ± SEM of three experiments with five mice per group. The significance of differences between the A⁺ and A⁻ groups is shown parenthetically. NS, not significant.

Much evidence suggests that vitamin A modulates gene expression, perhaps as a hormone-like molecule (1), thereby influencing cell differentiation. However, the precise mechanism for the vitamin's effects on gene expression and cell differentiation is unknown. If helper-T-cell differentiation or function is impaired by vitamin A deficiency, then antigen-driven helper-T-cell differentiation might provide an experimental model system in which to probe the biochemical mechanism for vitamin A's biological effects outside the visual cycle.

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